Secretory Overexpression of CGTase Gene from *Bacillus* sp. G1 in *Escherichia coli*

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Abstract

Cyclodextrin glucanotransferase (CGTases) catalyze the degradation of starch and maltodextrin to form cyclodextrins (CDs). CDs have various applications in food industry, cosmetic, pharmaceutical, toiletries, agrochemical industries and analytical chemistry. Overexpression of recombinant CGTase in intracellular *E. coli* expression system faces several problems especially the low expression level of recombinant CGTase and the formation of inclusion bodies. Low expression level is mainly due to the toxicity of accumulated recombinant protein that was expressed in the cytoplasm of *E. coli*, finally caused the cell death. Secretory *E. coli* overexpression system provides a promising solution to overcome this problem. The objective of this research is to overexpress the recombinant CGTase extracellularly in *E. coli* expression system. Preliminary studies show that co-expressing both CGTase and bacteriocin release protein (BRP) genes able to obtain the extracellular recombinant CGTase. The CGTase gene was amplified using polymerase chain reaction (PCR) method. The purified PCR fragment was cloned into pWH 1520 shuttle vector and transformed into *E. coli* that harbouring pJL3-BRP gene. The CGTase gene is regulated by *xylA* promoter and is induced by xylose; meanwhile the BRP gene is regulated by *lac* promoter and is induced by IPTG. The secretion pathway involves the permeabilization of the cell envelope that was activated by BRP and the release of bacteriocin molecules is accompanied by the release of the recombinant CGTase. The expression of extracellular recombinant CGTase at 37°C achieved 34.17 U/ml after 24 hours induction, with 0.5 mM xylose and 40μM IPTG as inducers.

Introduction

The overall objective in recombinant protein production is to simultaneously reach a high specific recombinant protein production rate, a high cell density and a high protein quality. Few expression systems have been developed to achieve more than one of these at a time. *Escherichia coli* expression system offers cheap, fast and convenient means for the production of recombinant protein. These advantages, coupled with a wealth biochemical and genetic engineering, have enabled the production of extracellular recombinant protein. Secretion of recombinant proteins to the culture medium of *E. coli* has several advantages over intracellular production. These advantages include simplified downstream processing, higher product stability and solubility, enhanced biological activity and N-terminal authenticity of the expressed peptide (Hannig and Makrides, 1998; Shokri *et al.*, 2003; Choi and Lee, 2004; Mergulhão *et al.*, 2005). Another advantage of extracellular secretion from *E. coli* is that because this bacterium does not normally secrete proteins into the culture medium, contamination of the product by the host proteins can be minimized (Shokri *et al.*, 2003; Mergulhão *et al.*, 2005).

Co-expression of bacteriocin release protein (BRP) provides an alternative approach to promote a limited leakage of the outer membrane for protein secretion. BRP also known as 'lysis proteins' or 'kil proteins', are involved in the transfer of colicins and cloacin DF13 from the *E. coli* cytoplasm to the culture medium (Wal. *et al.*, 1995). BRP is a small, 28-amino-acid, lipoprotein that is essential for the release of colicin into the medium, and activates the endogenous outer membrane phospholipase, which is important for the permeabilization of the cell envelope (Choi and Lee, 2004). BRP-mediated secretion of bacteriocins is semi-specific, because the release of bacteriocin molecules is accompanied by the release of a subset of cytoplasmic proteins and many periplasmic proteins (Wal *et al.*, 1995).

Cyclodextrin glucanotransferase (CGTase, 1,4, α-D-glucan; 1,4-α-D-glucopyranosyltransferase, EC 2.4.1.19) catalyze the degradation of starch and maltodextrin substrates to form cyclodextrins (CDs). CDs have various applications in food, pharmaceutical, cosmetic, toiletries, agrochemical industries and analytical chemistry. However, there are only a few reports with respect to the overexpression of recombinant CGTase in *E. coli*. Overexpression of recombinant CGTase inside the *E. coli* faces several problems especially the low expression level of recombinant CGTase and the formation of inclusion bodies. Low expression level is mainly due to the toxicity of accumulated recombinant protein that was expressed in the cytoplasm of *E. coli*. This problem can be overcome with designing a secretory *E. coli* expression system. The objective of this research is to over-express the extracellular recombinant CGTase with *E. coli* expression system, by co-expressing the BRP gene.

Materials and Methods

Bacterial strains and plasmids

The *cgt* gene was isolated from *Bacillus* sp. G1 (Ong, 2005). PCR method was applied to amplify the *cgt* gene with the restriction sites *BamH*I and *Sph*I added to the gene fragment. The forward primer used was synthesized based on the sequence starting from the mature gene (5'- tcg-gga-tcc-gac-gta-aca-aac-aaa-gta-aat-tac-3') and the reverse primer was designed based on the sequence of the termination codon (5'-tcg-gca-tgc-tta-cca-att-aat-cat-aac-cgt-atc-3'). The amplified PCR product was digested with restriction enzymes and was ligated with the pWH1520 shuttle vector (MoBiTec). The resulting plasmid was transformed into *E. coli* K12 N3406 [*Thr leu thi lacY tonA supE*] (MoBiTec) that carrying the pJL3-BRP gene by heat shock method (Sambrook *et al.*, 2001). Recombinant CGTase was expressed as extracellular enzyme by addition of 0.5mM xylose and 40μM isopropyl-b-D-thiogalactopyranoside (IPTG).

Expression of recombinant CGTase

To observe the recombinant CGTase secretion on the solid media, individual clones were spotted on the LB agar plates (Sambrook *et al.*, 2001) that were supplemented with 0.5% soluble starch, 100 μg/ml ampicilin and 0.5mM xylose. The plates were incubated at 37°C overnight and then the clones were induced by adding 20μl 40μM IPTG. The plates were incubated at 37°C overnight again and then stained with iodine solution to visualize the regions of starch digestion.

To analyze the recombinant CGTase secretion on the liquid media, individual clones were cultured at 37°C in modified TB medium (12g/l NZ Amine, 24g/l yeast extract, 8ml/l glycerol, 2.2g/l KH_2PO_4 and 9.4g/l K_2HPO_4). Aliquot of the bacterial culture liquid was removed and spun down after 24 post induction hours. The expression level of recombinant CGTase was determined by using CGTase assay.

CGTase Assay

The cyclization activity of CGTase was measured according to the Kaneko method (Kaneko *et al.*, 1987) with some modification. 1ml of 0.1M potassium phosphate buffer (pH 6.0) containing 40mg of soluble starch was mixed with 0.1ml of the supernatant or crude enzyme from the culture, and the mixture was incubated at 60°C for 10 minutes. Then the reaction was stopped by 3.5ml of 30mM NaOH. 0.5ml of 0.02% (w/v) phenolftalein in 5mM Na_2CO_3 was added to the mixture and mixed well. After leaving the mixture to stand for 15 minutes at room temperature, the reduction in colour intensity was measured at 550nm. A blank lacking the enzyme is tested simultaneously with each batch of samples. 1 unit of enzyme activity was defined as the amount of enzyme that forms 1 μmol of β-CD from soluble starch in one minute.

Result and Discussion

Effect of Co-expression Strategy on the Secretory Production

In order to express and secrete the recombinant CGTase from *E. coli* expression system, a co-expression strategy was applied. Bacteriocin release protein (BRP) gene was expressed together with the *cgt* gene, under different promoter regulation system. The *cgt*

gene was placed downstream of the *xyl* promoter in the recombinant plasmid pWH 1520 meanwhile the BRP gene was regulated by the *lac* promoter in the recombinant plasmid pJL3. To determine whether the recombinant CGTase could be secreted onto solid media, iodine staining method was applied to visualize the region of starch digestion. The colonies containing both genes clearly secreted the recombinant CGTase as evidenced by the halo of digested starch around the colonies (Figure 1). The formation of the halos resulting from starch digestion indicated that the secretory *E. coli* expression system was successfully achieved by co-expressing both BRP gene and *cgt* gene (Figure 1). In most cases, targeting the protein to the periplasmic space or to the culture medium facilitates downstream processing, folding and in vivo stability, enabling the production of soluble and biologically active proteins at a minimized processing cost (Mergulhão *et al.*, 2005). However, care must be exercised during such recombinant protein production so as not to over-induce the recombinant BRP, which will cause full-lysis and undesired cell death. Due to the potential quasi-lysis effect, co-expression must be carried out under a tightly repressible promoter and induction must be independent of the product gene promoter (Mergulhão *et al.*, 2005).

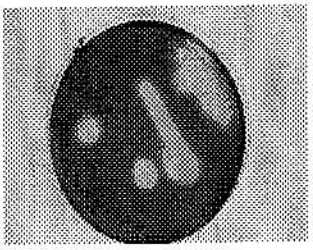

Figure 1: Extracellular Recombinant CGTase overexpressed by *E. coli* Expression System.

Effect of Co-expression Strategy on the Expression Level

Recombinant CGTase that is secreted as the extracellular protein was assayed to obtain the expression level after 24 post induction hours, and was detailed in Table 1. By referring to Table 1, CGTase excreted from the wild type *Bacillus* sp. G1 is 18.89 U/ml (Ho *et al.*, 2004). CGTase excreted from the wild type *Bacillus* sp. G1 after the medium optimization is 54.49 U/ml after 36 hours cultivation time (Ibrahim *et al.* 2005). The expression level at 37°C for the extracellular recombinant CGTase overexpressed by *E. coli* expression system is 34.17 U/ml after 24 post induction hours. The expression level of recombinant CGTase which is expected higher than the wild type was lower, after the medium was optimized for the production of CGTase in the wild type *Bacillus* sp. G1, This indicated that the optimization for the expression level of extracellular recombinant CGTase should be conducted to increase the production of recombinant CGTase extracellularly.

Wild type, *Bacillus* sp. G1		Recombinant clone, *E. coli*
CGTase activity, before medium optimization (U/ml)	CGTase activity, after medium optimization (U/ml)	CGTase activity (U/ml)
18.89*	54.49**	34.17

Table 1: Production of Extracellular CGTase by Wild Type and Recombinant Clone. *Ho *et al.* (2004), **Ibrahim *et al.* (2005)

Conclusion

In summary, by simply co-expressing *cgt* gene and BRP gene, a secretory *E. coli* expression system was successfully achieved. 34.17 U/ml recombinant CGTase was expressed after 24 hours post induction hours. To increase the expression level for the extracellular recombinant CGTase, optimization for the cultivation condition will be carried out.

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